Vascularisation for cardiac tissue engineering: the extracellular matrix

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Summary
Cardiovascular diseases present a major socio-economic burden. One major problem underlying most cardiovascular and congenital heart diseases is the irreversible loss of contractile heart muscle cells, the cardiomyocytes. To reverse damage incurred by myocardial infarction or by surgical correction of cardiac malformations, the loss of cardiac tissue with a thickness of a few millimetres needs to be compensated. A promising approach to this issue is cardiac tissue engineering. In this review we focus on the problem of in vitro vascularisation as implantation of cardiac patches consisting of more than three layers of cardiomyocytes (>100 µm thick) already results in necrosis. We explain the need for vascularisation and elaborate on the importance to include non-myocytes in order to generate functional vascularised cardiac tissue. We discuss the potential of extracellular matrix molecules in promoting vascularisation and introduce nephronectin as an example of a new promising candidate. Finally, we discuss current biomaterial-based approaches including micropatterning, electrospinning, 3D micro-manufacturing technology and porogens. Collectively, the current literature supports the notion that cardiac tissue engineering is a realistic option for future treatment of paediatric and adult patients with cardiac disease.

Keywords
Heart, vascularisation, coronary vasculature, tissue engineering, extracellular matrix

Introduction
Cardiovascular diseases (CVDs) present a major socio-economic burden in industrial countries and are a rising problem in low-income countries due to the increasing incidence of cardiac risk factors such as hypertension, obesity, and diabetes (1). Thus, major efforts have been invested aiming at the prevention of CVDs or the minimisation of cardiac damage (2–12). Unfortunately, despite significant advances the prevalence of heart failure has increased in the last decades (1). The World Health Organisation predicts that by 2030 more than 23 million people will die annually from CVDs. Moreover, congenital heart diseases (CHDs) are the most common type of birth defect affecting up to nine out of every 1,000 newborn babies (13). Therefore it is important to establish therapies to reverse cardiac damage incurred by CVDs and CHDs.

Loss of cardiomyocytes during cardiac disease
One major problem underlying most CVDs and CHDs is the irreversible loss of heart muscle cells, the cardiomyocytes. In a subset of cardiac diseases (e.g. myocardial infarction, cyanotic CHD) cardiomyocyte loss is due to ischaemia, which causes apoptosis, onclosis and necrosis of cardiomyocytes (14, 15). In addition, several congenital abnormalities such as abnormal development of the cardiac valves (perturbation of flow) or intrinsic genetic abnormality of the left ventricle result in the underdevelopment of the left ventricle (Hypoplastic Left Heart Syndrome) (16). Moreover, corrective surgical procedures of CHDs may result in cardiomyocyte loss. Finally, pathological remodelling induced by cardiomyocyte loss or anatomic as well as haemodynamic abnormalities causes further cardiomyocyte loss (17–19). As a consequence heart failure is reduced resulting in a diminished quality of life and inevitable progression to heart failure (Figure 1A). This is an increasing issue in CHD patients. Surgical procedures have been significantly improved, thus resulting in a rapidly expanding population of adults who underwent palliative surgery for CHD in infancy or childhood. These patients are at risk for heart failure due to chronic pressure and/or volume loading caused by possibly remaining residual anatomic and haemodynamic abnormalities, inadequate myocardial preservation during prior surgeries, myocardial fibrosis, surgical injury to a coronary artery, and/or pulmonary hypertension (20, 21).
Cardiac disease and vascularisation. A) Causes of cardiomyocyte loss leading to heart failure. B) Thebesian veins. Three distinct forms of these veins are known, which drain blood directly from the capillary, from the arteries without crossing the capillary, or from the coronary veins. C) Cardiac tissue patches require an adaptor to connect their capillary bed to a host artery for proper blood circulation after transplantation. Transplantation on top of the epicardium requires access to an artery and a vein, while transplantation on the endocardium or a transmural insertion might not require a connection to the host venous system considering that drainage of venous blood direct into the ventricular cavity is feasible.

The complexity of the heart

The heart is a highly complex organ consisting of a large variety of cell types. While cardiomyocytes account for around 75% of the heart volume, more than 40% of the cells in the heart are non-myocytes (e.g. fibroblasts, endothelial cells, vascular smooth muscle cells, pericytes, neurons, perineurial cells, and Schwann cells [47–51]). In addition, the heart does not contain one but several different types of cardiomyocytes that are of different developmental origin and exhibit different characteristics in regards to their contractility and their electrophysiological properties (52–54). This variety in cell types allows the formation of a complex four-chambered heart regulated by an electrical system, the cardiac conduction system, which consists of the sinoatrial node, the atrioventricular (AV) node and the His-Purkinje system. The different cardiomyocytes and their orientation result in a helical heart, that pumps in a torsional fashion (55, 56). In order to perform coordinated contractions the heart requires a stress-tolerant, viscoelastic extracellular matrix (57). Consequently, the generation of a genuine piece of myocardium is a great challenge (32, 44).

Given the complexity of the heart it appears unlikely that adding only cardiomyocytes to the heart is sufficient to improve heart function. It rather seems that an effective regenerative therapy needs to introduce the proper myocardial cell types at the right place in the right orientation into the heart. For instance, the implantation of cardiomyocytes with sinus node conduction properties into the working myocardium might lead to life-threatening arrhythmias. However, several publications suggest that the addition of only cardiomyocytes is able to significantly improve cardiac function. In 2003, Reffelmann et al. critically evaluated whether cardiomyocyte transplantation is a valid approach to treat heart failure. They concluded that transplanted foetal and neonatal car-
Cardiomyocytes can functionally integrate and enhance cardiac function (58). In 2005, Pasumarthi et al. showed that cardiomyocyte-specific overexpression of cyclin D2 induces cardiomyocyte proliferation and can regenerate the heart after cardiac injury (59). These data suggest that proliferating cardiomyocytes themselves can induce proliferation, migration and differentiation of other cell types required to form new cardiac tissue. Finally, Zimmermann et al. demonstrated in 2006 that implantation of so-called EHTs (engineered heart tissue) made only from isolated neonatal rat heart cells and collagen type I from rat tails significantly improved heart function after experimental myocardial infarction in immune-suppressed rats. Analysis after 28 days showed no evidence of arrhythmia. Importantly, implantation prevented further dilation, induced systolic wall thickening of infarcted myocardial segments and improved fractional area shortening (60). Thus, one promising approach to treat heart diseases appears to be tissue engineering, which allows the generation of tailor-made cardiac patches containing the right cells in the right orientation in the right viscoelastic scaffold.

The importance of non-myocytes

Although several studies have indicated that transplantation of cardiomyocytes can improve heart function, there is evidence that addition of other heart cells can increase the beneficial effect of cardiac patches. It has been demonstrated that cardiomyocyte proliferation plays an important role in natural occurring cardiac regeneration in zebrafish and newborn mice (61, 62). Studying these model systems has, however, also demonstrated that factors released from endothelial cells are required for the restoration of the cardiac tissue (63–65). Already a decade ago it had been shown that overexpression of vascular endothelial growth factor (VEGF) in early-differentiated cells from mouse embryonic stem cells significantly enhanced their ability to improve heart function in a mouse infarct model (66). The possibly beneficial effects of including non-myocytes in the generation of cardiac patches have been demonstrated in 2006 when Naito et al. optimised the EHT technology. EHTs made from a mix of cardiomyocytes and non-myocytes developed three times higher forces than those made from purified cardiomyocytes (67). The beneficial effect has subsequently been confirmed in several other approaches utilising primary or stem cell-derived cardiomyocytes together with fibroblasts and/or endothelial cells resulting in improved tissue structure and function (68–71). Caspi et al. showed that the presence of embryonic fibroblasts stabilise the vessel-like structures in engineered cardiac tissue (72). However, it is poorly understood how fibroblasts and endothelial cells promote cardiac tissue formation. Newman et al. analysed the contribution of fibroblast to angiogenesis in an in vitro system. Their data indicate that fibroblasts produce extracellular matrix (ECM) proteins that are essential for endothelial cell lumen formation (73). In addition, Zeng et al. have suggested that fibroblast-produced ECM stimulates growth and metabolism of cardiomyocytes at least in vitro (74).

The need for vascularisation in cardiac tissue engineering

The human heart beats 60 to 80 times per minute at a basal rate and has a very high metabolic activity that requires an extensive vascularisation (2,400–3,300 capillaries/mm², intercapillary distance of around 19–20 µm) (75, 76). Myocardial infarction causes severe damage to the human left ventricular wall, which is around 10 mm thick (77). Thus, to replace the lost myocardium cardiac patches might need to be generated that are several millimetres thick.

Recently, it has been demonstrated that subcutaneous implantation of non-vascularised cardiac patches consisting of more than three layers of cardiomyocytes in nude rats results in necrosis. However, cell sheets of three layers were vascularised in vivo within one to two days. Repeated transplantation of cardiomyocyte cell sheets on top of each other allowed the generation of around 1 mm thick cardiac patches with a well-organised microvascular network (Figure 2A) (78). Furthermore, it has been shown that cell sheets can directly be transplanted onto the heart (79). However, this “multi-step transplantation” approach will be difficult to translate into clinical practice. An attempt to improve the cell sheet technology is the combined transplantation with omentum that consists of connective tissue, fat, a rich vasculature, and lymphatic vessels and that contains angiogenic factors. Omental transplantation is a well-established surgical procedure to promote organ repair (80, 81). Kawamura et al. have demonstrated that covering cell sheets of human induced pluripotent stem cells (hiPSC)-derived cardiomyocytes with omentum allowed the generation of a stable seven-layered cardiac patch (82). Collectively, these data suggest that the generation of cardiac patches of relevant thickness critically depends on an efficient in vitro vascularisation procedure.

Vascularisation

Vascularisation occurs through two processes: vasculogenesis and angiogenesis (83). Vasculogenesis is defined as the de novo formation of blood vessels from endothelial progenitor cells (EPCs). In the murine yolk sac, these EPCs migrate and associate to form discrete blood islands. A subset of these cells, the angioblasts, proliferate and undergo further differentiation into endothelial cells that form by fusion primitive tube-like vessels; a vascular plexus (84). Similarly two decades ago experimental data from chick-quail chimeras demonstrated that the coronary vasculature forms from a plexus-like vascular network. Cells of the proepicardium migrate and attach to the looped heart and proliferate forming the epicardium. A subpopulation of epicardial cells undergoes epithelial-mesenchymal transformation, migrates into the subepicardial and myocardial layer and produce ECM components. Subsequently, these mesenchymal cells give rise to cardiac fibroblasts, coronary vascular smooth muscle, and coronary endothelial cells. The subepicardial endothelial cells coalesce to form a vascular plexus. Subsequently, the endothelial tubes mature in
larger vessels by the recruitment of pericytes, smooth muscle cells (SMCs), and fibroblasts (85, 86). Recent lineage tracing experiments suggest that subepicardial endothelial cells are the major source of intramyocardial coronary arteries in the ventricular wall, and that coronary arteries and veins have a common origin in the developing heart (87). Coronary circulation is established by anastomosis with the base of the aorta.

Angiogenesis describes the process of vascular expansion by sprouting of capillaries from pre-existing blood vessels. Upon proangiogenic stimuli endothelial cells differentiate into tip cells, which express proteolytic enzymes that remodel cell-cell junctions. This allows tip cells to migrate in response to guidance signals. Neighbouring endothelial cells, the so-called stalk cells, start to proliferate to form a lumen and to elongate the sprout, which is

Figure 2: Cardiac tissue engineering. Examples of techniques used in cardiac tissue revascularisation. For details see text and references.
stabilised by pericyte recruitment. Upon fusion of neighbouring sprouts, endothelial cells become quiescent phalanx cells, which are covered by mature pericytes and deposit new basal membrane to establish a barrier resulting in vascular expansion (88–90). Angiogenesis plays an important role in physiology and pathophysiology. In diseases like cancer or inflammation, the goal is to inhibit vascularisation, while in ischaemic diseases the goal is to enhance vascularisation (83, 90, 91).

It is important to recognise that the vasculature is a complex network of blood vessels that significantly differ in length, diameter, composition and function. There are three major types of blood vessels. Arteries, which carry the blood away from the heart (inside diameter in the mm range, aorta: 25 mm [92]), branch and narrow into arterioles (93), and then further branch into capillaries (5 µm inside diameter [94]). Capillaries connect arterioles and venules (7–50 µm) enabling the actual exchange of gases, sugars, nutrients and waste chemical substances between blood and tissues. After tissue perfusion the capillaries join into wider venules, which in turn further widen and converge to become veins, which carry blood from the capillaries back to the right atrium through the coronary sinus. Both in veins and arteries, the vessel wall is comprised of three layers: a) tunica intima – innermost layer, which consists of endothelial cells, basement membrane and connective tissue; b) tunica media – middle layer (thickest in arteries), which consists of smooth muscle cells and collagen fibres; c) tunica adventitia – outermost layer (thickest in veins), which consists of loose connective tissue (93). Capillaries consist of little more than a layer of endothelium and occasional connective tissue (94).

In the arterial system, the blood pressure is usually around 120 mm Hg systolic (high pressure wave due to contraction of the heart) and 80 mmHg diastolic (low pressure wave). In contrast, pressures in the venous system are constant and rarely exceed 10 mm Hg. Thus the tunica media of arteries carry more smooth muscle cell layers and elastin fibres than veins to strengthen the vessel wall. Largest arteries like aorta, pulmonary trunk, and common carotid arteries carry around 40 to 70 layers and smallest arteries carry around 20 layers of smooth muscle cells, separated by elastin fibres (95). Arterioles, the smallest of the true arteries, have the greatest collective influence on both local blood flow and on overall blood pressure by the variable contraction of the smooth muscle of their walls. In contrast, veins have a significantly thinner wall, thus they are more flexible and provide a “volume reservoir” (96).

Another important difference between vessels is the structure of the inner endothelial lining. Endothelial cells can provide an uninterrupted lining ensuring that only smaller molecules, such as water and ions are able to diffuse in the tissue (97). In contrast, so-called fenestrated capillaries have pores allowing also a limited amount of protein to diffuse (98). For example, non-continuous lining can be found in the renal glomerulus. Here, specialised endothelial cells, the podocytes, have foot processes, which have slit pores (99). Finally, sinusoidal capillaries have the largest openings in the endothelium, which allow red and white blood cells and various serum proteins to pass (100).

Coronary vasculature

The heart muscle is supplied with oxygenated blood through the coronary arteries (101). The right and left main coronary arteries branch off the ascending aorta just above the aortic valve. The left main coronary artery splits into the circumflex artery and the left anterior descending artery, which supplies the left ventricle. The coronary arteries branch into arterioles and these arterioles then branch into innumerable capillaries that deliver oxygenated blood to the transmural myocardium. Finally, the blood is collected through venules that coalesce into the cardiac veins and is returned to the right atrium through the coronary sinus, where it joins the systemic deoxygenated blood entering from the superior and inferior venae cavae. In addition, Adam Christian Thebesius discovered venous drainage into the ventricular cavity. Three distinct forms of these veins, called Thebesian veins, are known. They drain blood directly from the capillary, from the arteries without crossing the capillary, or from the coronary veins (►Figure 1B). Under physiological conditions these veins contain 5%-10% of venous return. However, if the epicardial coronary veins are compromised they can drain the majority of venous return (102).

A particularity of the coronary blood flow is due to the cross-talk between the myocardium and the coronary vasculature (102, 103). Coronary blood flow depends on the interrelation of four factors: aortic pressure, myocardial extravascular compression (the myocardial contraction during the systole nearly stops coronary inflow), myocardial metabolism (local control), and neural control.

Considering the anatomy of the coronary vasculature and the need for vascularisation in cardiac tissue engineering, a capillary bed inside a 3D construct has to be generated. In addition, an adaptor is needed to connect the capillary bed to a host artery for proper blood circulation after transplantation. The necessity of a connection to a host vein depends on the transplantation site of the cardiac patch. While the transplantation on top of the epicardium requires such a connection, transplantation on the endocardium or a transmural insertion might not require a connection to the host venous system considering that drainage of venous blood direct into the ventricular cavity is feasible (►Figure 1C).

Current approaches to improve vascularisation in cardiac tissue engineering

The use of scaffolds is common practice in cardiology. In paediatric cardiology scaffolds are required to correct congenital heart defects (104). But also for adult patients an acellular approach might be beneficial, as scaffold implantation can improve the flexibility and stability of the ventricular wall (105, 106). However, these approaches do not restore the lost cardiac tissue and in children the implants cannot adapt to natural organ growth, and thus require re-operation (104).

Endogenous cardiac stem cells have been discovered in animal models (33, 107, 108). Moreover, a clinical study indicated that endogenous stem cells could be utilised to repair the damaged
human heart (35). In addition, it has been suggested that at least a sub-population of human cardiomyocytes retain the ability to proliferate (43). As adult zebrafish and newts can regenerate their heart by cardiomyocyte proliferation many laboratories try to find ways to efficiently induce mammalian cardiomyocyte proliferation (41, 42). Therefore, major efforts are invested in acellular devices that contain cues to enhance endogenous cardiac repair. However, it is controversial whether it is possible to harness endogenous regenerative mechanisms of the heart (42, 45, 46, 109).

Here, we focus on the generation of tailor-made functional heart tissue patches \textit{ex vivo} by combining cells, matrices, biological active molecules and physiologic stimuli (110). Such cardiac patches have several purposes: i) restoration of cardiac function after surgical repair (correction of congenital heart defects) (104, 111, 112); ii) use as \textit{in vitro} model systems (e.g. screening platform to determine drug toxicity) (113); iii) cardiac valve replacement (112, 114, 115); iv) transplantation of \textit{in vitro} engineered 3D cardiac tissue in order to improve adult cardiac function after injury (44).

Cardiac patches made from cardiomyocytes with or without endothelial cells develop a primitive vascular network that participates in the process of \textit{in vivo} vascularisation (67, 116–118). However, \textit{in vitro} these structures do not supply oxygen and nutrients and they cannot be directly connected to the host circulation. Thus, the thickness of viable cardiac tissue \textit{in vitro} in the absence of directed perfusion is limited to 50 to 100 µm (78, 119), which can be enhanced by perfusion and high oxygen up to around 200 µm (120). As patches of a few millimetres thickness are needed to reverse damage incurred by myocardial infarction or surgical corrections of cardiac malformations, it is mandatory to enhance \textit{in vitro} vascularisation and/or to improve cell survival under hypoxic conditions (121).

One of the first approaches to improve perfusion was to mimic a vasculature by electrosprinning of micro- and nano-fibrous scaffolds (Figure 2B) (122). This idea has continuously improved, and has recently resulted in lithographic-generated endothelialised microfluidic vessels within a native collagen matrix (123), the fabrication of a silk-based construct containing a network of microchannels surrounded by a porous scaffold (124) and carbon-nanotube reinforced bioprintable vascular conduits (125). Whether such channels can indeed act as vascular bed allowing the generation of large tissue patches has still to be proven. However, Baranski et al. have recently generated engineered vessels using a microtissue molding (Figure 2C) approach, which integrated with host blood vessels as early as three days after implantation and progressively matured for 28 days (126).

Another approach to generate a vascular network is to utilise the endogenous vasculature of the host. Pre-implantation of a scaffold \textit{in vivo} will result in its vascularisation. After explantation the vascularised patch can be seeded with cardiac cells (127–129). A more advanced approach is to implant a chamber with cells and a matrix in the host. An arteriovenous loop is placed in the chamber, which is subsequently filled with cells and scaffold material. This approach results in a well-vascularised cardiac patch with a defined arterial and venous access for implantation (130). However, such approaches might be difficult to translate from bench to bedside. Therefore, it is important to establish an \textit{in vitro} vascularisation model. Recently, Sekine et al. have demonstrated that isolated rat grown tissue can act as vascular bed \textit{in vitro}. Artery and vein were connected to a medium perfusion system, which allowed vascularisation of cardiac cell sheets produced from co-culture with endothelial cells (131). Chiu et al. utilised two blood vessel explants, which they fixed to opposite sides of a collagen-chitosan hydrogel. To promote the formation of a vascular bed they embedded thymosin β4. In addition, they utilised micropatterned silicone surfaces to direct the outgrowth of the capillaries from the vessels (132). A recent study observed that alginate matrices containing microchannels of 200 µm diameter combined with adhesion peptides and angiogenic growth factors promoted formation of vessel-like networks, both \textit{in vitro} and \textit{in vivo} (133).

Human umbilical vascular endothelial cells (HUVECs) were first seeded and cultured for three days, followed by seeding with neonatal rat cardiomyocytes (CMs) and cardiofibroblasts. Constructs were implanted subcutaneously in mice, and higher extent of cell penetration as well as greater vessel density was found in scaffolds containing microchannels. Clearly, application of innovative manufacturing approaches to create anisotropic 3D structures exhibiting porous (channel-like) morphology and matching mechanical properties are approaching the goal of having functional patches with superior performance for cardiac regeneration. However, surface conditioning by incorporating specific signalling molecules is an essential requirement to enhance the vascularisation of such artificial matrices (134, 135).

Growth factors play an important role in the formation of capillary networks, which has extensively been reviewed (136–144). In brief, VEGF, fibroblast growth factors (FGFs), and Notch signalling control endothelial cell behaviour, activation and tip/stalk cell selection, while the subsequent recruitment and differentiation of perivascular cells or smooth muscle cells from the surrounding stroma, to promote vessel tightness and stabilisation, is regulated by platelet-derived growth factor (PDGF) and transforming growth factor beta (TGFβ). Finally, angiopoietins (ANGs) regulate vessel tightness, whereby ANG1 stimulates mural coverage and basement membrane deposition while ANG2 mural cell detachment enabling endothelial cell sprouting and angiogenesis. VEGF, FGFs, Notch, and ANGs play key roles also during coronary vasculature development. Accordingly, these factors have been proposed as novel candidates for therapeutic revascularisation in ischaemic heart disease (85, 145–147). However, despite encouraging preclinical data, clinical studies have not yet led to commercial approval (148).

A major issue in applying growth factors is that they have to be provided in an ordered sequence for a specific time and concentration (84, 149). For example, VEGF, considered the master determinant of vasculogenesis and angiogenesis, is a promising factor to promote angiogenesis in a clinical setting. However, initial experiments utilising long-term constitutive expression of VEGF resulted in abnormal blood vessels and hemangiomas. Recently, it has been demonstrated that morphology and function of VEGF-induced blood vessels depend on VEGF dosage (150). An ap-
proach to simulate the role of the ECM in development to control
the release of growth factors is the field of modular tissue engin-
eering. Tiruvannamalai-Annamalai et al. have recently utilised this
system to engineer liver tissue by using biodegradable microcap-
sules with tunable interior environments. The principle is to en-
capsule functional cells and seed endothelial cells on the surface of
the capsules (Figure 2D). Fusion of these capsules resulted in
3D constructs with an embedded network of interconnected chan-
els that enabled long-term perfusion culture of the construct
(151).

Other innovative methods to generate artificial scaffolds that mimic
the natural ECM are micropatterning, soft lithography techniques (Figure 2E), and 3D printing technology (Figure 2F) (152–155). Studies on these methods have revealed that the
topography of scaffolds is important for cell attachment and behav-
iour. Madden et al. have for example shown that in poly(2-hy-
droxyethyl methacrylate-co-methacrylic acid) hydrogel-based
scaffolds a channel diameter of 60 µm was necessary to promote
cardiomyocyte attachment and aggregation. In addition, pore sizes
of 30–40 µm allowed angiogenesis and reduced fibrotic response
(155). A broad range of biocompatible, namely synthetic and natu-
ral polymers as well as their blends are being considered to develop
cardiac patches (156, 157). Recent developments have focused on a
particular class of elastomeric polymers, such as polyglycerol seba-
cate (PGS) (158), sometimes combined with natural polymers
such as gelatine or collagen. They are processed in a variety of
morphologies, which include nano-fibrous scaffolds fabricated by
electrospinning (154, 159) and 3D structures fabricated by laser
microablation (Figure 2G) methods (153) combined with
microelectromechanical systems (MEMS) fabrication and packag-
ing techniques (Figure 2H) (160). It is important to generate a
scaffold with the proper mechanical characteristics exhibiting the
intrinsic anisotropy and directionality of the host tissue. In this
case, scaffolds with an accordion-like honeycomb structure
have been generated that mimic the native human myocardium
(153). In a study by Radisic et al. (135), scaffolds were fabricated
with the ability to provide oxygen supply to cells placed within
PGS constructs, both myocytes and non-myocytes (fibroblasts). To
mimic the capillary network, a highly porous PGS scaffold was
used in which parallel arrays of channels of 377 ± 52 µm in diam-
eter were introduced (135). To perfuse pre-vascularised cardiac
patches perfusion bioreactors have been developed (68). The pa-
rameters of perfusion should be adjusted in a way that the result-
ing shear stress stabilises the existing capillary bed (161–163).
Collectively, none of the current approaches allows yet the gen-
eration of a sufficiently thick cardiac patch due to the lack of a ma-
ture and stable vascularisation. However, significant advances have
been made that make the generation of a pre-vascularised cardiac
patch that can be connected to the coronary circulation a realistic
future option to treat cardiac diseases.

Bottlenecks in cardiac tissue engineering

A tissue engineering approach appears promising, as engineered
tissues such as trachea (164), blood vessels (165), and urinary
bladder (166) have already been utilised in the clinical setting. Ac-
cordingly, a large number of studies have attempted to engineer
scaffold-based cardiac tissues (44, 156). As described above it has
been demonstrated that implantation of cardiac engineered tissue
can improve cardiac function in small animal models such as the
rat (60). However, the translation into the clinical setting is ham-
pered by several issues (44, 110). The human left ventricle contains
around 2 to 3 billion cardiomyocytes (167). A myocardial infarct,
one of the leading causes of death (1), is in most patients lethal if
more than 30% of the left ventricular free wall is affected (168,
169), which equals around 750 million cardiomyocytes. Thus, to
improve cardiac function after myocardial infarction, tissue
patches containing several 100 million cardiomyocytes need to be
generated. It was long unclear how to solve this issue, as no clini-
cally applicable cell type was available to generate cardiomyocytes
in a large scale. The importance of this problem and advances in
the field of stem cell biology (170) resulted in an increasing effi-
ciency of commercial large-scale productions of cardiomyocytes
from hiPSC (e.g. axiogenesis). The hope is that autologous hiPSC
could be produced to avoid immune rejection. However, it re-
mains unclear if hiPSC-derived cells elicit immune rejections and
the production of autologous hiPSC is time consuming and cost-
intensive (171–174). A possible solution might be the establish-
ment of a bank containing several hiPSC lines that offer human
leukocyte antigen-matched products minimising the need for im-
mune suppression (44). An alternative cell source might be the use
of parthenogenetic stem cells (175). Additional issues that need to
be solved regarding hiPSC or stem cells in general are genomic al-
terations due to prolonged in vitro culturing and/or the process
of reprogramming (176–180) and the lack of protocols that promote
differentiation beyond a foetal/neonatal-like to an adult cardio-
myocyte phenotype (181, 182). The immature phenotype of stem-
cell-derived cardiomyocytes can cause arrhythmias and limits the
contractility of the cardiac patch (ranging between 0.1 and 4 mN/
mm² [183] vs 50 mN/mm² of human explanted adult cardiac tis-
sue [184]). Besides the proper cell source the successful translation
of cardiac tissue engineering into the clinical setting requires also
an immune compatible scaffold.

Finally, in order to significantly improve cardiac function by
implanting a pre-vascularised cardiac patch, the capillary network
needs to remain stable and functional after implantation in the pa-
tient, anastomosis to the host vascularisation must be stable allow-
ing normal blood flow, and thrombosis must be prevented. Simi-
larly to the problem of finding an appropriate resource for cardio-
myocytes, it remains a challenge to obtain cells to generate a capil-
ary network available in an abundant number, which are well tol-
erated by the host’s immune system. Autologous vascular cells can
be harvested from patient blood vessels, but only in limited
numbers (185). A solution to this problem might be an expansion
of autologous endothelial progenitor cells ex vivo as previously
tested by Atluri et al. utilising stromal cell-derived factor-1 (186).
Alternative sources of cells are stem cells that can be differentiated into vascular cell types such as endothelial progenitor cells or iPSC (185, 187–189). Recently, Pagliari et al. have for example generated pre-vascularised cardiac tissue based on porous gelatine scaffolds utilising human mesenchymal stem cells to generate endothelial cells and cardiomyocyte progenitor cells (163).

As described above, capillaries are stabilised by pericytes that share with the endothelial cells a basal membrane (190, 191). Thus, just seeding endothelial cells together with cardiomyocytes will not be sufficient to generate a vascularised cardiac patch. Instead, it will be important to add also pericytes or stem cells as well as growth factors, to promote the formation of an interconnected capillary structure. Once a functional stable capillary network has been established the problem of connecting the microscopic capillaries with the macroscopic arterial and venous blood vessels in the host has to be solved. Due to the enormous oxygen consumption of cardiac muscle the cardiac patch must be directly connected to the host circulation to prevent necrosis. Thus, cardiac patches should be designed in such a way (e.g. containing vascular pedicles or engineered equivalents) that they can be sutured by a surgeon and anastomosed to the appropriate artery and vein in the recipient site. In the heart it is possible to take advantage of a long history of bypass surgery, in which atherosclerosis-free vein and/or artery grafts taken from another area of the body are used to replace atherosclerotic vessels. However, bypass surgeries also revealed additional hurdles that need to be overcome. Major problems are for example shear stress due to flow disturbances at the end-to-side graft–artery configuration resulting in endothelial damage/dysfunction leading to proliferation and overgrowth of smooth muscle, and/or thrombosis and finally stenosis of the vessel (192, 193).

The inner lumen of the circulatory system is covered with endothelial cells, which inhibit immunogenic reactions and thrombosis as well as smooth muscle cell proliferation (190, 191). The need for endothelisation or the use of non-immunogenic material is a significant problem in coronary stent technologies. Thus, major efforts are devoted to identify stent coating or platform materials that demonstrate excellent endothelial-cell supportive and non-thrombogenic properties (194). Such materials might be useful to create artificial capillary beds for cardiac tissue patches. Otherwise, it will be necessary to generate a continuous epithelialised microvasculature. Strategies to improve in situ endothelialisation of biodegradable polymeric grafts include chemical and physical modifications to graft surfaces in order to for example enhance the recruitment of endothelial or endothelial progenitor cells (195). Recently, a living microfluidic vascular network made of endothelialised microchannels was successfully established (123). Additional complications can be calcification and graft rupture (165, 196).

The potential of the ECM in regenerative medicine and tissue engineering

Historically, the ECM was primarily viewed as a supporting inert scaffold. The ECM is a heterogeneous mixture of water, saccharides, and various protein components traditionally classified into four categories: collagens, proteoglycans, non-collagenous glycoproteins, and elastins. In recent years it has been shown that the ECM is not only a physical framework. It is now well established that the ECM is required for angiogenesis (197). The cancer field has significantly contributed to our understanding of the mechanisms promoting angiogenesis and recognised that the tumour microenvironment, meaning the crosstalk between different cell types and the ECM, plays an essential role (198). Already in 1993, George et al. provided the first genetic evidence for a major role of the ECM in vascular development. They showed that fibronectin knockout mice were embryonic lethal exhibiting severe cardiovascular defects (199). Subsequently, several independent laboratories demonstrated by gene ablation in mice that several other ECM components are required for proper vascularisation during development (reviewed in [200]). The ECM plays a role in vascularisation also in humans, as it is exemplified by the Marfan Syndrome. This connective tissue disorder is caused by fibrillin-1 mutations that cause vascular abnormalities due to perturbations of TGF-β1 signalling (54).

The importance of the ECM has been demonstrated by studying natural occurring regeneration. Unlike mammals, adult lower vertebrates like newt and zebrafish can regenerate after injury (201). In newts, ECM components like fibronectin, tenascin-c and hyaluronic acid play a crucial role in limb regeneration (202, 203). Very recently, Mercer et al. demonstrated that fibronectin, tenascin-C, and hyaluronic acid are also significantly up-regulated during newt cardiac regeneration. Their data suggest that these ECM components are positive inducer for the accumulation and migration of cardiac progenitors (204). The requirement for fibronectin in cardiac regeneration has recently demonstrated in zebrafish. Fibronectin became up-regulated in the epicardial layer as soon as one day after myocardial injury. Importantly, lack of fibronectin in fibronectin mutant zebrafish directly inhibits cardiomyocyte proliferation after myocardial injury (205). Thus, a detailed knowledge about the role of ECM components in natural occurring regeneration might provide important clues for the establishment of regenerative therapies.

The potential of the ECM in medicine is exemplified by the early observations that, given the appropriate cues, aggressive carcinoma cells can be tamed to form normal tissues or can be reverted to a normal phenotype (206). The following examples demonstrate the importance of understanding ECM function for regenerative medicine: i) The ECM can regulate the fate of stem cells (207). ii) Cells do not only detect the composition and stiffness of the ECM but they can also detect its geometry. This is true also for artificial scaffolds: pore sizes in engineered trabecular bone, for example, depend on the initial scaffold geometry (208). iii) Xenogeneic (i.e. porcine) ECM has been successfully used as a biologic scaffold for esophageal reconstruction in a dog model.
When the ECM alone was used as a template for reconstruction for partial-circumference esophageal defects, the healing response showed organised layers of muscle, submucosal tissue, and an intact mucosal layer (209). 4) It has been shown that chondroitin sulphate proteoglycans are the principal inhibitory component of glial scars, which form after damage to the adult central nervous system and act as a barrier to regenerating axons. Importantly, administration of chondroitinase ABC, an enzyme that selectively cleaves GAG chains from the chondroitin sulphate proteoglycans, promotes the regeneration of sensory and corticospinal tract axons after lesion (210). 5) Manipulation of single components of the ECM can improve cardiac tissue repair (211). 6) Administration of a fragment of the ECM protein perlecan, domain V, 24 hours after stroke is neuroprotective and proangiogenic (212).

Decellularised tissue: xenogenic ECM for regenerative medicine

The realisation that the ECM encodes important cues for cell behaviour and organ development raised the question whether decellularised tissues/organs could be used as platforms for tissue engineering and regenerative medicine (213–215). Decellularisation is achieved by perfusion of allogeneic or xenogeneic tissues employing osmotic shock, ionic and non-ionic detergents, proteolitic digestions and DNase/RNase treatments to remove all cells generating a cell-free ECM (216). Optimally, these scaffolds retain their 3D anatomical architecture including acellular vascular conduits, their physiochemical properties as well as glycosaminoglycans (GAGs) and growth factors to provide the necessary cues during the re-cellularisation process for cell attachment, differentiation, vascularisation, and function.

The use of ECM has already been translated into the clinic. There is for example a lot of experience in the use of bovine and allogenic ECM to correct congenital cardiac anomalies in paediatric patients (217–219). Recently, Faulk et al. have reviewed the use of ECM scaffolds (prepared from tissues such as dermis, heart, urinary bladder, small intestine, mesothelium, and pericardium) from a variety of species including human, porcine, bovine and equine. These scaffolds have for example been utilised in the clinic to reconstruct a variety of tissues including skin (chronic wound), urinary bladder, rotator cuff (214) and airway (164).

The potential of the ECM for cardiac tissue engineering has been highlighted in 2008 by decellularisation and re-seeding experiments on whole rat hearts. Ott et al. perfused the heart with 1% SDS and reseeded the decellularised hearts with rat aortic endothelial cells and neonatal cardiomyocytes. The observation that the reseeded constructs exhibited only a pump function equivalent to about 2% of an adult heart indicated that this approach needs to be significantly improved (220). Nevertheless, this work supported the idea that decellularised xenogeneic organs might be the solution to cardiac tissue engineering.

Wainwright et al. improved the decellularisation process as 1% SDS resulted in increased stiffness of the ECM. They demonstrated that serial perfusion with an enzymatic, non-ionic detergent, ionic detergent, and acid solution with hypotonic and hypertonic rinses allows efficient decellularisation of porcine hearts maintaining physicochemical properties comparable to the native heart (221). Aubin et al. have recently further improved the decellularisation procedure by introducing a software-controlled automated coronary perfusion (222). These studies have demonstrated that the cardiac ECM contains all instructional cues to allow functional recovery after infusion or intramural injection of a suspension of functional cells. An alternative to decellularisation of hearts is the use of autologous omentum-based matrices. Shevach et al. recently demonstrated that cardiac cells could assemble in these matrices into elongated and aligned tissues (223). In addition, when endothelial cells were utilised blood vessel networks were formed in these patches.

The approach to decellularise organs faces several problems that have not yet been solved. One issue is that decellularised organs are thrombogenic. Thus it is important to seed endothelial cells to generate a continuous endothelial layer inside the vasculature before re-cellularisation with cardiac cells (224). Recently, it has been demonstrated that decellularised mouse hearts can be repopulated with iPSC-derived cardiovascular progenitor cells. Importantly, the cells migrated, proliferated and differentiated in situ into cardiomyocytes, smooth muscle cells and endothelial cells. However, even though re-cellularised scaffolds exhibited spontaneous contractions, generated mechanical force and were responsive to drugs, they contained regions of uncoupled tissue, which is concurrent with non-homogenous connexin 43 expression patterns (225). These studies have also indicated that the repopulation of such a complex organ structure is difficult. However, even if the scaffolds can efficiently be repopulated, problems such as stable excitation-propagation and proper alignment of cardiomyocytes remain (226).

Although the field of organ decellularisation is rather young, significant progress has been made. Considering the above-described issues it remains unclear if it will be indeed possible to generate complex tissue-engineered organs like the heart but the generation of cardiac patches or the use of large vessels based on xenogeneic scaffolds appears to be a realistic future option. Thus, it is important to consider the immunogenicity of allogeneic and xenogeneic biological scaffold materials. While xenotransplantation of intact organs cause severe immune reactions and poses the risk of transmission of infectious agents, the use of ECM derived from allogeneic or xenogeneic tissues appears safe and is already clinically applied (215). However, it is important to ensure that the decellularised scaffolds do not retain cellular debris and thus the cell surface antigen Galα1-3Galβ1-4GlcNAc-R (Gal) (227). The Gal epitope is absent in humans and apes and is implicated in the hyperacute rejection phenomena following xenogeneic whole-organ xenotransplantation (228). In contrast, ECM scaffolds derived from porcine tissue do not elicit significant immune reactions, although the Gal epitope could be detected in small amounts in the decellularised scaffolds. Consequently, the host response to both wild-type and Gal-deficient xenogeneic scaffold materials were not significantly different (229). Finally, it has been indicated that remaining viral DNA (e.g. porcine endogenous retrovirus
(PERV)) if present in the ECM could not transmit the disease to a human host (230).

Collectively, the use of decellularised tissue/organs provides a promising platform for tissue engineering and regenerative medicine.

The role of ECM in vascularisation

Blood vessels consist mainly of ECM proteins that provide vessels with different physicochemical properties. More than 100 ECM proteins have been reported including collagens, elastin, fibronectin, fibrillins, abundant amorphous or soluble proteoglycans, and leucine-rich glycoproteins (231, 232). These ECM proteins are expressed by the different vascular cells. Smooth muscle cells for example produce in the media collagen and elastin, while fibroblasts in the adventitia produce collagen, osteopontin, and fibronectin (232, 233).

The ECM regulates cell adhesion, cell survival, proliferation, migration, and differentiation for example by modulating the activity, bioavailability, or presentation of growth factors to cell surface receptors (234). Moreover, it plays an essential role in morphogenesis, tissue maintenance, tissue repair and disease (213, 235–237). The fact that endothelial cells secrete extracellular macromolecules that play an important role in basal lamina formation and at the same time influence the endothelial cell behaviour was suggested more than three decades ago (238). Meanwhile it has been proven that the ECM regulates also in the vessel wall cell adhesion, migration, proliferation, and phenotype (232).

One way in which the ECM regulates vascular cell behaviour is through a direct interaction of their RGD domains with matrix receptors such as integrins on the cell surface, which allows the cells to react to changes in matrix rigidity and composition (200, 239, 240). That the composition of the ECM is essential became clear by the observation that basal lamina proteins collagen-IV, laminin, and perlecan limit smooth muscle cell proliferation and enhance contractile gene expression while interstitial matrix proteins collagen-I, collagen-III, fibronectin, and osteopontin enhance smooth muscle cell proliferation (241). The analysis of individual ECM components has provided important insights in their effect on vascular cells and thus their potential use in cardiac tissue engineering. For example, elastin preserves the quiescent, contractile phenotype of smooth muscle cells (242, 243) and collagen regulates via both β1 integrin and the discoidin-domain receptor family members (244) the cellular behaviour of smooth muscle cells (e.g. α1β1 integrin, and the discoidin-domain receptor family members (244) the cellular behaviour of smooth muscle cells (e.g. α1β1 integrin, proliferation [245]), endothelial cells (e.g. αvβ3 integrin, adhesion [246]), as well as fibroblast (collagen-IV, differentiation; collagen-I and collagen-III [247]). A detailed review on this topic has recently been published by Xu et al. (232).

Besides the direct ECM-cell interaction, the ECM utilises several other mechanisms to regulate angiogenesis. First, growth factors such as VEGF bind to the ECM. The activity of the growth factors depends on their affinity to the ECM, which can be altered post-translationally either by proteolytic processing of the growth factors (248) or modulation of the ECM itself. For example, the sulphation pattern of heparan sulphate proteoglycans fine-tunes the complex FGF signalling networks (249–251). Second, due to its ability to bind growth factors the ECM significantly contributes to the establishment of growth factor gradients. Third, it has been shown that partial proteolysis of ECM macromolecules releases peptides, so-called matrikines, which modulate cell behaviour and many of which display anti-angiogenic activities (91, 234, 252, 253). Fourth, the ECM regulates angiogenesis based on its stiffness (254).

The use of the ECM in cardiac tissue engineering

In order to generate a vascularised tissue it is possible to utilise ECM components. Several ECM proteins have been shown to be pro-angiogenic. For example, Gerstel et al. have shown that carcinomaembryonic antigen-related cell adhesion molecule 1 (CEA-CAM1) contributes to tumour angiogenesis and vascular maturation in mammary carcinomas (255). The data by Yang and Yee suggest that the V2 isoform of the proteoglycan versican plays a pro-angiogenic role in glioblastomas, one of most angiogenic human tumours. Fibronectin was up-regulated by V2 and mediated V2 function (256). Recombinant peristin and its fasciclin I domain have been shown to induce neovascularisation in a murine model of limb ischaemia (257). Recently, Ciucurel et al. utilised the ECM protein Developmental endothelial locus-1 (Del-1) to enhance a modular approach to generate a large vascularised bed. They transduced the endothelial cells that coat the modules with a lentiviral construct to overexpress Del-1 (258). However, there have also been efforts to use minimal structures (just RGD motif for functionalisation) to mimic the ECM. For example, Mammadov et al. developed a self-assembling peptide amphiphile molecule that is functionalised with biologically active groups to mimic heparin (259). Grant et al. demonstrated that a synthetic peptide from the α1 chain of Laminin (SIKVAV) promotes angiogenesis (260), which was verified by several other investigators (261).

The use of individual ECM components is a promising start in enhancing scaffolds by promoting angiogenesis. However, the experience with decellularised tissues raises the question whether it is possible to generate an artificial ECM as scaffold for cardiac tissue engineering. Decellularisation of hearts is complicated by several issues such as complete removal of cells while maintaining ECM surface topography and at the same time retaining resident ligands (214). In addition, decellularised hearts cannot be tailored. Thus, it would be desirable to manufacture an artificial ECM in vitro to generate ECM scaffolds for specific applications in a standardised and reproducible way. A first step in this direction is the development of bioinks for the bioprinting of cell-laden constructs by solubilisation of decellularised tissues (262). However, in the future it will be important to elucidate the composition of the target organ ECM and to better understand the role of the individual components. In addition, these components need to be generated as recombinant proteins that are processable. Recently, we have identified nephronectin as an important regulator of en-
dothelial cell differentiation during atroventricular valve development in zebrafish (263). Nephroectin is an ECM protein containing EGF-like repeats, an RGD sequence and a C-terminal MAM domain (264). In a subsequent study, we determined that nephroectin exhibits excellent adhesive properties to promote adhesion of cardiomyocytes, cardiac endothelial cells as well as fibroblasts (265). Our data suggested that nephroectin has the potential to improve current approaches to generate cardiac patches by promoting cardiomyocyte attachment and vascularisation. This hypothesis was supported by the observation that the related protein EGFL7, which like nephroectin is a secreted protein containing EGF-like repeats and an RGD sequence, could enhance vessel formation (266). In the future it will thus be important to investigate whether nephroectin plays a role in vessel formation in vivo during development and disease and to assess its ability to promote or stabilise vascularisation in cardiac tissue engineering.

**Conclusion**

Cardiac tissue engineering is a promising approach to treat CVDs and CHDs. The surgical repair of complex congenital heart defects frequently requires additional tissue in various forms, such as patches, conduits, and valves. These devices often require replacement over a patient’s lifetime because of degeneration, calcification, or lack of growth. The main new technologies in congenital cardiac surgery aim at, on the one hand, avoiding such reoperations and, on the other hand, improving long-term outcomes of devices used to repair or replace diseased structural malformations (112). These technologies are: 1) new patches: CorMatrix® patches made of decellularised porcine small intestinal submucous extra-cellular matrix; 2) new devices: the Melody® valve (for percutaneous pulmonary valve implantation) and tissue-engineered valved conduits (either decellularised scaffolds or polymeric scaffolds); and 3) new emerging fields, such as antenatal corrective cardiac surgery or robotically assisted congenital cardiac surgical procedures. These new technologies for structural malformation surgery are still in their infancy but certainly hold great promise for the future. However the translation of these emerging technologies to routine health care and public health policy will also largely depend on economic considerations, value judgments, and political factors (112).

Recent studies have demonstrated that it is possible to generate a cardiac patch utilising (stem cell-derived) cardiomyocytes, endothelial cells and fibroblasts. However, the issue of vascularisation and thus the generation of a thick functional cardiac patch have not yet been solved. Thus, in the future it will be important to identify scaffold materials (single or blended) that allow the attachment of these cell types and the establishment and stabilisation of a vasculature. In addition, these scaffolds have to be bio-/immune-compatible and to possess mechanical properties allowing optimal force generation and elasticity. A promising approach appears to be the use of 3D printing technology and micropatterning to generate a basis for a vascular bed, which can be seeded/printed with endothelial cells, and a surrounding matrix to seed/print cardiomyocytes and fibroblasts. In contrast to the current approach to manufacture a scaffold and subsequently seed cells, which is a major problem in thick 3D structures, 3D printing technology allows precise placement of cells and biomolecules within a confined 3D structure (267). However, several issues have to be overcome before it will be possible to print a cardiac patch such as the lack of mechanical strength and integrity as well as the print resolution (jetting-based: 50 μm; extrusion-based: 100–300 μm). An important step has been recently made by Kolesky et al. who developed a large-area 3D bioprinter with four independently controlled printheads to print pre-vascularised constructs based on multiple types of cells and ECM (268). Alternatively, the different cell types could be encapsulated in so-called porogens of gelatine, alginate and hyaluronic acid, which can be degraded in response to specific stimuli including temperature, chelating and enzymatic digestion (269). To improve the biological performance of cardiac patches pro-angiogenic ECM molecules or cytokines/growth factors should be incorporated. Finally, the vascular bed should have an entrance and exit that can first be used as access points for a medium perfusion system and afterwards for the connection to the host vascularisation. An alternative could be the use of xenogeneic decellularised vessels that do not elicit an immune rejection.

**Figure 3**: Suggested approach to generate thick vascularised cardiac tissue patches to treat heart disease. 3D printing technology allows printing cells in a scaffold in the right configuration utilising several different print-materials (e.g. natural or synthetic functionalised with growth factors or protein domains such as RGD peptides) optimised for the different cell types (cardiomyocytes, endothelial cells, pericytes, fibroblasts). The vascular bed should have an entrance and exit that can first be used as access points for a medium perfusion system and afterwards for the connection to the host vascularisation. An alternative to print vessels could be the use of xenogeneic decellularised vessels that do not elicit an immune rejection.
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Conflicts of interest
None declared.

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